Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 371 (2007) 154–161
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Bioluminescence immunoassay for angiotensin II using aequorin as a label

Xiaoge Qu [a], Sapna K. Deo [a], Emre Dikici [a], Mark Ensor [a], Michael Poon [b], Sylvia Daunert [a,*]

[a] Department of Chemistry, University of Kentucky, Lexington, KY 40506, U.S.A.
[b] Zena and Wiener Cardiovascular Institute, Mount Sinai School of Medicine, New York, NY 10029, U.S.A.

Received 15 May 2007
Available online 1 September 2007

Abstract

Angiotensin II is a biologically active component of the renin–angiotensin system. High levels of angiotensin II may be responsible for hypertension and heart failure because they increase systemic vascular resistance, arterial pressure, and sodium and fluid retention. Therefore, it is important to monitor angiotensin II levels for the treatment of hypertension and heart diseases. The goal of this work was to develop a bioluminescence immunoassay using aequorin as a label to measure angiotensin II levels in human plasma. This method utilizes a genetically engineered fusion protein between angiotensin II and aequorin. For that, the C terminus of angiotensin II was fused to the N terminus of apoaequorin using molecular biology techniques. A heterogeneous immunoassay was then developed for the determination of angiotensin II. A detection limit of 1 pg/mL was obtained with the optimized assay, allowing for the determination of angiotensin II at physiological levels in human plasma.

Keywords: Bioluminescence immunoassay; Aequorin; Angiotensin II; Reporter protein

The renin–angiotensin system plays vital roles in regulating blood pressure, blood volume, and electrolyte homeostasis. Renin, secreted by the kidneys, cleaves angiotensingen to yield the decapeptide angiotensin I. Angiotensin I is then cleaved further into the octapeptide angiotensin II by the action of the angiotensin-converting enzyme. Angiotensin II is short lived and is degraded into angiotensin III and inactive products. It causes vasoconstriction and increases arterial pressure by binding to angiotensin II type 1 receptors. Moreover, angiotensin II stimulates the secretion of aldosterone, which causes an increase in sodium levels and fluid retention [1–5]. All of these events can cause hypertension, left ventricular hypertrophy, and heart failure [6,7]. Therefore, it is important to monitor the levels of angiotensin II in plasma for the treatment of hypertension and heart diseases. Due to the low concentration of angiotensin II in plasma, methods with increased sensitivity are required to measure angiotensin II. Conventional methods for the determination of angiotensin II in plasma are based on radioimmunoassay (RIA)[1] [8–11]. Although RIA methods have good sensitivity, they are not satisfactory because these methods require the use of the radioactive isotope ^{125}I, which may cause health hazards and waste disposal problems. On the other hand, RIA is often coupled with high-performance liquid chromatography [9,11,12] so that a pretreatment of the plasma sample is usually needed to extract, purify, and concentrate the angiotensin II. Yasunaga *et al.* [13] in 1992 reported an enzyme immunoassay (EIA) method

* Corresponding author. Fax: +1 859 323 1069.
E-mail address: daunert@uky.edu (S. Daunert).

[1] *Abbreviation used*: RIA, radioimmunoassay; EIA, enzyme immunoassay; AEQ, aequorin; IPTG, Isopropyl β-thiogalactopyranoside; BSA, bovine serum albumin; DTT, dithiothreitol; PBS, Phosphate buffered saline; TBS, Tris-buffered saline; CBD, chitin binding domain.

0003-2697/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.08.038

based on the fluorescence emission when β-D-galactosidase reacted with 4-methylumbelliferyl β-D-galactopyranoside. Volland *et al.* [14] developed an EIA method based on the solid-phase immobilized epitope immunoassay technique for the determination of angiotensin II. Although these methods did not need radioisotopes, they involved extensive pretreatment of the sample. Methods based on capillary electrophoresis with UV detection have also been reported. Lacher *et al.* [15] used the on-capillary copper (II) complexation method to determine angiotensin peptides without any extraction steps. The sensitivity of this system was low and not appropriate for angiotensin II measurement in human plasma. On the other hand, Sim and Qui [16] were able to obtain good sensitivity, but the method involved laborious pretreatment of the sample, namely, a series of extraction and preconcentration steps. Therefore, a simple, rapid, and sensitive method without pretreatment of plasma would be advantageous for the determination of angiotensin II in plasma. To that end, our work focused on the development of an immunoassay for angiotensin II using the bioluminescent protein aequorin (AEQ) as a label. The assay demonstrated high sensitivity, and low detection limits and required no pretreatment of the sample.

Bioluminescence is a rare natural phenomenon in which light is generated by a biochemical reaction. Aequorin is a bioluminescent protein, originally isolated from the jellyfish *Aequorea victoria* [17]. The photoprotein is composed of apoaequorin (189 amino acids), coelenterazine (a chromophore), and molecular oxygen. Aequorin has three Ca^{2+}-binding EF-hand regions and undergoes a conformational change upon binding of Ca^{2+}. This results in the oxidation of coelenterazine to coelenteramide accompanied by production of CO_2 and an emission of blue light ($\lambda_{max} \approx$ 469 nm) [18]. This process allows for the detection of aequorin down to attomole levels because of the low background signal [19]. Given the intense light emission afforded by this process, this photoprotein has been employed in several biological and analytical applications, such as intracellular calcium detection [20,21], immunoassays, and highly sensitive binding assays [19,22,23]. In our laboratory, we have employed native and genetically modified aequorin as a label for the detection of small biological molecules. Among these, we have developed bioluminescence assays for the measurement of thyroxine [24], leuenkephalin [25], prostacyclin [26], and cortisol [27] in biological fluids.

In this work, we aimed to develop a bioluminescence immunoassay for angiotensin II using aequorin as a label. To achieve this goal, we constructed a fusion protein between the angiotensin II DNA sequence and the gene sequence for a cysteine-free aequorin (AEQ mutant S). For that, a plasmid containing the DNA sequence encoding for the fusion protein was constructed. The plasmid was transformed into *Escherichia coli* cells, and the protein was expressed. After purifying the fusion protein, a heterogeneous immunoassay was developed for angiotensin II using microtiter plates. The optimized immunoassay was employed to measure angiotensin II concentration in human serum.

Materials and methods

Materials

Tris (hydroxymethyl) amino methane (Tris)-free base was purchased from Research Organics, Inc (Cleveland, OH). Isopropyl β-thiogalactopyranoside (IPTG) was obtained from Gold Biotechnology (St. Louis, MO). Luria Bertani (LB) broth was obtained from Becton–Dickinson (Sparks, MD). Ethylenediaminetetraacetic acid (EDTA) sodium salt, glucose, agar, sodium dodecyl sulfate (SDS), sodium phosphate, Tween 20, bovine serum albumin (BSA) and all other reagents were purchased from Sigma (St. Louis, MO). All enzymes were obtained from New England Biolabs (Beverly, MA). All primers used for polymerase chain reaction (PCR) were purchased from Operon Biotechnologies (Huntsville, AL). Reacti-bind 96-well neutravidin-coated white polystyrene microtiter plates were from Pierce (Rockford, IL). Coelenterazine was obtained from Biotium (Hayward, CA). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). The IMPACT-TWIN kit for affinity purification of fusion protein was purchased from New England Biolabs. Biotinylated goat anti-rabbit F_c-specific secondary antibody and rabbit anti-human angiotensin II primary antibody were from Chemicon (Temecula, CA) and Phoenix Pharmaceuticals (Belmont, CA), respectively. Angiotensin II peptide (human) was obtained from Sigma. All chemicals used were reagent grade or better, and water used to prepare solutions was deionized (Milli-Q water purification system, Millipore, Billerica, MA) distilled water.

All buffer solutions used were as follows: buffer 1 (20 mM Tris containing 1 mM EDTA, 500 mM NaCl, pH 7.0), buffer 2 (20 mM Tris containing 2 mM EDTA, 1000 mM NaCl, 0.1% Tween 20, 1 mM DTT, pH 7.0), buffer 3 (20 mM Tris containing 1 mM EDTA, 40 mM DTT, 500 mM NaCl, 0.1% Tween 20, pH 8.5), dialysis buffer (30 mM Tris containing 2 mM EDTA, pH 7.4), PBS blocking buffer (100 mM sodium phosphate, 150 mM NaCl, 0.05% Tween 20, 0.1% BSA, pH 7.4), PBS wash buffer (100 mM sodium phosphate containing 150 mM NaCl, 0.05% Tween 20, pH 7.4), TBS blocking buffer (30 mM Tris containing 150 mM NaCl, 2 mM EDTA, 0.05% Tween 20, 0.1% BSA, pH 7.4), and TBS wash buffer (30 mM Tris containing 150 mM NaCl, 2 mM EDTA, 0.05% Tween 20, pH 7.4), and luminescence triggering buffer (100 mM Tris–HCl, 100 mM $CaCl_2$, pH 7.4).

Apparatus

PCRs were performed using a Pelkin–Elmer Gene Amp PCR system 2400 (Norwalk, CT). The cells were collected with a Beckman centrifuge. Cells were lysed by a 550 sonic